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A new gibberellin detection system in living cells based on antibody V_H/V_L interaction

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ABSTRACT

As a new detection method of bioactive gibberellin A_4 (GA_4) in living cells, a combined system of GA_4-dependent interaction of V_H and V_L composed of a variable region fragment (Fv) of anti-GA_4 antibodies and protein-fragment complementation assay (PCA) was developed. First, when V_H and V_L were displayed in proximity on a phage, they could constitute a functional Fv. Thereafter, V_H and V_L were shown to interact with each other in a GA_4-dependent manner. We then applied this interaction to PCA using GFP as a reporter. V_H fused to the C-terminal half of GFP and V_L fused to the N-terminal half of GFP were simultaneously expressed in *Escherichia coli*. The *E. coli* in which these fusion proteins were inductively produced in the presence of GA_4 showed clear GFP fluorescence, while those in the absence of GA_4 showed only scarce GFP fluorescence, demonstrating the feasibility of this system to detect GA_4 in living organisms.

Gibberellins (GAs) are essential phytohormones involved in many aspects of plant development such as seed germination, stem elongation, and the development of flowers, fruits, and seeds. Detailed analysis of endogenous GAs has offered fundamental information, e.g., in determining the major biosynthetic pathway in each plant species, demonstrating the blocking step in GA biosynthetic mutants, demonstrating the feedback- and feedforward-regulation of GA biosynthetic and catabolizing enzyme genes, etc. Analytical methods of endogenous phytohormones include instrumental analyses such as GC/MS and LC/MS/MS, and immunological analyses such as enzyme-linked immunosorbent assay (ELISA) and radio immunoassay (RIA). Although instrumental analyses are broadly used due to the high sensitivity and identification reliability, expensive devices and sometimes special technique are required. Contrarily, immunological analyses demand no special devices and are easy to handle. Antibodies against GAs have been used for immunoassays, not only as an alternative method to instrumental analyses, but as an alternative to bioassays for monitoring fractions containing GAs reactive to these antibodies. The capacity of these antibodies to detect GAs in situ, i.e., immunological detection of GAs in tissue or organs, has also been examined. The general problem in detecting small molecules in situ is the difficulty in fixing them. Hasegawa et al. reported the detection of GA_4 in rice anthers by fixing GAs with 1,3-diisopropylcarbodiimide gas after rapidly freezing them with liquid nitrogen [1]. Although they solved the problem of fixation, their method was not applicable to other plant materials because of the low sensitivity or low signal/noise ratio; rice pollen was an extraordinarily rich source of GA_4.

To detect GAs in situ, a new detection system independent of GA fixation to tissue is required. We assumed that two protein fragments which associate with each other in a GA-dependent manner can be used to this end, since various methods to detect protein–protein interaction have been extensively developed such as protein-fragment complementation assay (PCA) [2] and fluorescence resonance energy transfer (FRET) [3]. PCA and FRET have already been applied in plant research for demonstrating the association of two fragments *in planta* [4].

In the general PCA strategy, any reporter protein can be rationally dissected into two fragments, which are fused to two probe proteins that are thought to bind to each other. Re-constitution of the reporter protein is catalyzed by the binding of the probe proteins. Variable region fragment (Fv) is the binding domain of immunoglobulin, which is composed of a variable region domain of heavy and light chains (V_H and V_L, respectively). In this study,

Abbreviations: GA, gibberellin; GFP, green fluorescent protein; ELISA, enzyme-linked immunosorbent assay; V_H, variable fragment of H chain; V_L, variable fragment of L chain; Fv, variable fragment; scFv, single-chain Fv.

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V_H and V_L of anti-bioactive GA antibodies were examined as candidates of two probe proteins which interact with each other in a GA-dependent manner. Ligand-dependent interaction of V_H and V_L was found by Ueda et al. [5] and has been used for establishing non-competitive ELISA called open-sandwich ELISA (OS-ELISA), to analyze ligand with higher sensitivity compared with the conventional competitive ELISA [6]. We first confirmed the GA_4-dependent interaction of V_H and V_L fragments from two independent anti-GA_4 antibodies, and then examined the application of those fragments to PCA using GFP to detect GA_4 in *Escherichia coli* as a simple living organism.

Materials and methods

Vector construction, protein expression and ELISA analyses by the split-Fv system. DNA fragments corresponding to V_H and V_L regions of anti-GA_4 monoclonal antibodies 8/E9 and 21/D13 were PCR amplified with the respective plasmids, pHEN2-F6 and pHEN2-D11(E2G) [7], with the primer sets for incorporating NcoI and XhoI sites in V_H fragment and SalI and NotI sites in the V_L fragment (for 8/E9-V_H: fw, 5′-cgg*ccatgg*[NcoI]gggtgaagctggtggagtc-3′; rv, 5′-gg*ct cgag*[XhoI]gagacggtgacagaggttc-3′, for 8/E9-V_L: fw, 5′-cc*gtcgac* [SalI]tgtgatgacccaaaccccactc-3′; rv, 5′-tg*cggccgc*[NotI]ccgttttatttc caactttgtccc-3′, for 21/D13-V_H: fw, 5′-cgg*ccatgg*[NcoI]gggtgaagc tggtggagtc-3′; rv, 5′-gg*ctcgag*[XhoI]gagacggtgaccgtgg-3′, for 21/D13-V_L: fw, 5′-cc*gtcgac*[SalI]aagcatgttgctgactcagtctcc-3′; rv, 5′-tg*c ggccgc*[NotI]ccgttttatttccaactttgtccc-3′). These fragments were digested with the incorporated restriction sites and cloned into the same sites of pKST2 developed for the split-Fv system [7]. Each expression vector that was obtained was introduced into *E. coli* strain TG1 and HB2151 cells by electroporation and separately subjected to preparation of phages simultaneously displaying V_H and V_L, or phage-displayed V_H and soluble V_L, as described in [7]. The interaction of GA_4 and Fv displayed on phage was detected by ELISA according to our previous method [6] except that BSA-GA_4 was coated on ELISA wells at 12.5 μg/mL in PBS. The GA_4-dependent interaction of soluble V_L and phage-displayed V_H was assayed by OS-ELISA following the method of Aburatani et al. [8], in which various concentrations of GA_4 (up to 0.1 mM) were examined.

Production of soluble V_L as a fusion with glutathione-S-transferase (GST), and phage-displayed V_H as a fusion with p3. The V_L gene fragment was excised from the above prepared vectors in pKST2 with SalI and NotI and ligated into the same restriction sites in pGEX-4T-2 (GE Healthcare, Uppsala, Sweden). The resulting vector was transformed into *E. coli* host strain Rosetta (Merck Biosciences, Darmstadt, Germany) and incubated in 10-mL LB medium containing 2% glucose, 34 μg/mL chloramphenicol, and 100 μg/mL ampicillin until $OD_{600} = 0.6$. The *E. coli* was pelleted, re-suspended with 10-mL LB containing 1 mM IPTG in addition to chloramphenicol and ampicillin, and incubated at 25 °C for 12 h. The bacterial pellet was sonicated in PBS containing 1 mg/mL lysozyme, and the supernatant after centrifugation at 3300*g* for 10 min was recovered as a soluble fraction. After confirming the recovery of GST-V_L in the soluble fraction by SDS–PAGE and CBB staining, GST-V_L was purified by affinity chromatography using Glutathione–Sepharose 4B (GE Healthcare) according to the manufacturer's protocol, resulting in 360 μL of GST-V_L fraction.

DNA fragments corresponding to V_H regions of 8/E9 and 21/D13 were PCR amplified with the respective plasmids, pHEN2-F6 and pHEN2-D11(E2G) [7], with the primer sets for incorporating NcoI and NotI sites (for 8/E9-V_H: fw, 5′-cgg*ccatgg*[NcoI]gggt gaagctggtggagtc-3′; rv, 5′-cc*gcggccgc*[NotI]ggagacggtgacagaggttc-3′, for 21/D13-V_H: fw, 5′-cgg*ccatgg*[NcoI]gggtgaagctggtggagtc-3′; rv, 5′-cc*gc ggccgc*[NotI]ggagacggtgaccgtgg-3′). These fragments were digested with the incorporated restriction sites and cloned into the same sites of pHEN2 [9], a phagemid vector for displaying p3 fusion proteins. Phages displaying V_H were prepared from 20-mL culture according to our previous method [7] and recovered in 1 mL PBS.

OS-ELISA with phage-displayed V_H and GST-V_L. Each well of ELISA plates (Nunc maxisorp immuno plate, 96 wells, Thermo Fisher Scientific) was coated with 100 μL of anti-GST antibody (GE Healthcare) diluted 1000 times with 50 mM $NaHCO_3$ (pH 9.6) overnight at 4 °C. Before reaction of GST-V_L with the coated anti-GST antibody, GST-V_L (20 μL of the above affinity-purified fraction) and phage-V_H (20 μL of the above preparation) were incubated with or without 10 μM GA_4 in 100 μL of 2% skimmed milk in PBS (MPBS) for 1 h at ambient temperature. The mixture was added to the wells after blocking with MPBS for 2 h, and incubated for 2 h. The wells were washed with PBS containing 0.1% (v/v) Tween 20 and then with PBS alone, and the bound phages were detected with the horseradish peroxidase (HRP)-conjugated anti-M13 antibody (5000 times diluted with PBS, GE Healthcare, Uppsala, Sweden) and subsequent reaction by adding 100 μL of a substrate solution [100 mM sodium acetate (pH 6) containing 3,3′,5,5′-tetramethylbenzidine (100 μg/mL) and hydrogen peroxide (0.6% (v/v))]. The enzyme reaction was stopped by adding 50 μL of 1 M H_2SO_4. Peroxidase activity was evaluated by calculating the difference in absorbance (A) at 630 and 450 nm ($A_{450} - A_{630}$).

PCA based on GA_4-dependent interaction of V_H and V_L of 8/E9. Because we tried some expression systems to detect GA_4-dependent V_H/V_L interaction, the procedure of construction of the expression vector for PCA was not straightforward. All PCR amplifications of V_H or V_L and NGFP or CGFP below were performed respectively with pHEN2-F6 [7] or CaMV35S-sGFP(S65T)-NOS3′ [10] as plasmid templates. First a vector to express V_H-NGFP fusion protein was constructed in pGEX-4T-2 (GE Healthcare). A DNA fragment for the V_H region was amplified using a primer set for incorporating BamHI and EcoRI sites: fw, 5′-cc*ggatcc*[BamHI]atgggggtgaagctgg-3′; rv, 5′-cc*gaattc*[EcoRI]caagcttggagacggtgacagaggttc-3′. NGFP region was amplified using a primer set: fw, 5′-cc*gaattc*[EcoRI]tgggtggc tccggtggctccggcggg*tcgac*[SalI]catggtgagcaagggcg-3′; rv, 5′-cc*gcg gccgc*[NotI]ctgcttgtcggccatgatatagac-3′. The 5′ region between EcoRI and SalI sites of the forward primer encoded a short linker peptide. The V_H and NGFP regions were cloned into the BamHI/EcoRI sites and EcoRI/NotI sites of pGET-4T-2, respectively, giving pGEX-V_H-NGFP. The V_H and NGFP fragments in pGEX-V_H-NGFP were respectively replaced with NGFP and V_L obtained by PCR using primers (for NGFP: fw, 5′-cc*ggatcc*[BamHI]atggtgagcaagggc gag g-3′; rv, 5′-cc*gaattc*[EcoRI]tctgcttgtcggccatgatatagac-3′, for V_L: fw, 5′-cc*gtcgac*[SalI]tgtgatgacccaaaccccactc-3′; rv, 5′-tg*cggccgc*[NotI]ccgttttatttccaactttgtccc-3), yielding pGEX-NGFP-V_L. On the other hand, the NGFP fragments in pGEX-V_H-NGFP were replaced with CGFP obtained by PCR using primers: fw, 5′-cc*gtcgac* [SalI]gaa gaacggcatcaaggtgaacttc-3′; rv, 5′-cc*gcggccgc*[NotI]cttgtacagctcgtcc atgcc-3′, yielding pGEX-V_H-CGFP. With pGEX-V_H-CGFP as a template, V_H-CGFP fragment was amplified using primers: fw, 5′-cc*ggatcc*[BamHI]ggatgggggtgaagctggtggagtc-3′; rv, 5′-cc*gcggc cgc*[NotI]cttgtacagctcgtccatgcc-3′. With pGEX-NGFP-V_L as a template, NGFP-V_L fragment was amplified using primers: fw, 5′-ggca tatg[NdeI]gtgagcaagggcgagg-3′; rv, 5′-ggctcgag[XhoI]ccgttttattt ccaactttgtccc-3′. The above V_H-CGFP and NGFP-V_L fragments were, respectively, cloned into BamHI/NotI and NdeI/XhoI sites of pETDuet-1 (Merck Biosciences). The obtained plasmid vector was transformed into *E. coli* host strain Rosetta-gami (Merck Biosciences). Expression of NGFP-V_L and V_H-CGFP was induced as mentioned above for GST-V_L preparation. GFP fluorescence was analyzed under a fluorescence microscope (BX60, Olympus) with either a U-MWIB/GFP or a U-MNIBA filter (Olympus).

Results

Separately produced V_H and V_L fragments can assemble to bind GA_4

In this study, V_H and V_L fragments of two independent monoclonal antibodies against GA_4, designated as 8/E9 and 21/D13, were used. These antibodies specifically recognize the structures characteristic of bioactive GAs [7,11]. We had also prepared single-chain antibody (scFv), a recombinant form of binding domain in which V_H and V_L are connected with a flexible linker peptide, using gene fragments encoding those domains and showed that the binding property was similar to the original antibodies [12]. Before examining the GA_4-dependent interaction of V_H and V_L, we confirmed that the separately produced V_H and V_L can assemble to form Fv and showed its binding activity to GA_4. The assay was performed by the phage-based "split-Fv system" developed by Aburatani et al. [8], which can be used both for detecting the binding activity of Fv to antigen, and for monitoring the ligand-dependent association of V_H and V_L, depending on *E. coli* host strains for the expression. In an amber-suppressor strain such as TG1 strain, V_H and V_L are expressed as fusions with p9 and p7 coat proteins, respectively, thereby displayed in proximity on the phage surface. Thus V_H and V_L constrained to be close are supposed to easily assemble to form binding domain and show binding activity to antigen. Contrarily, in a non-suppressing strain such as HB2151, V_L is produced as a soluble protein fused with a c-myc tag while V_H is displayed on the phage. By fixing V_L by anti-c-myc antibody or protein L coated on an ELISA plate, the ligand-induced interaction of V_L and V_H can be detected. When V_H/V_L of anti-GA_4 antibodies were expressed by the former expression system, the binding activity to BSA-GA_4, the original immunogen of the antibodies, was clearly detected both for 8/E9 and 21/D13 (Fig. 1). The binding was outcompeted by an excess amount of free GA_4, indicating the interaction of Fv reconstituted by V_H and V_L with the GA_4 moiety. Then, the GA_4-dependent interaction of V_H and V_L was examined using V_H displayed on phage and a soluble form of V_L fused with a c-myc epitope tag, expressed in a non-suppressing strain. However, the expected interaction was not detected (data not shown). Western analysis of V_H and V_L revealed that the amount of V_L in the culture medium is below the detection level, while V_H was successfully detected as a fusion with p9 (data not shown), indicating the low stability/productivity of the V_L fragment in this expression system.

V_H and V_L assemble in GA_4-dependent manner

Since V_L was shown to be unstable in the above expression system, a more general expression method of recombinant proteins was examined, i.e., V_L was expressed as a fusion with glutathione-*S*-transferase (GST) under a *tac* promoter. The protein was successfully produced as a soluble protein and then the interaction of the affinity-purified GST-V_L with V_H was examined. V_H was displayed on phage by expressing as a fusion with p3, which is the most common expression system for phage display. First, we confirmed that the phage-V_H and GST-V_L can form an Fv domain and recognize GA_4, even if V_H and V_L are expressed separately and not in proximity, unlike the expression system in Fig. 1. It was shown that the phage displaying V_H could bind BSA-GA_4 in the presence of GST-V_L and that this binding was inhibited by excess GA_4 (Fig. 2). Binding was not observed when GST-V_L was excluded from the reaction mixture, showing that V_H and V_L assembled to form a correct F_V domain with binding activity. Finally OS-ELISA to demonstrate the ligand-dependent interaction of V_H and V_L was performed as in Fig. 3A. GST-V_L incubated with phage-V_H either in the presence or absence of GA_4 was captured by anti-GST antibody adsorbed on an ELISA plate surface. The amount of bound phage clearly increased by the addition of GA_4 especially when V_H and V_L of 8/E9 Ab was used (Fig. 3B), while those of 21/D13 showed modest GA_4-dependent interaction. Because the absorbance (A_{450}-A_{630}) was about 0.01 when either anti-GST Ab or GST-V_L was excluded from the reaction procedure (data not shown), the observed absorbance level in the absence of GA_4 was likely to show a GA_4-independent interaction of phage-V_H and GST-V_L.

GA_4 can be detected by GA_4-mediated PCA using GFP as a reporter

The applicability of GA_4-dependent V_H/V_L interaction to the GA_4 monitoring system based on PCA was examined using V_H and V_L of 8/E9, which showed a clearer GA_4-dependent interaction than with 21/D13. GFP was dissected into an N-terminal half (NGFP) and a C-terminal half (CGFP) between residues 157 and 158, a position

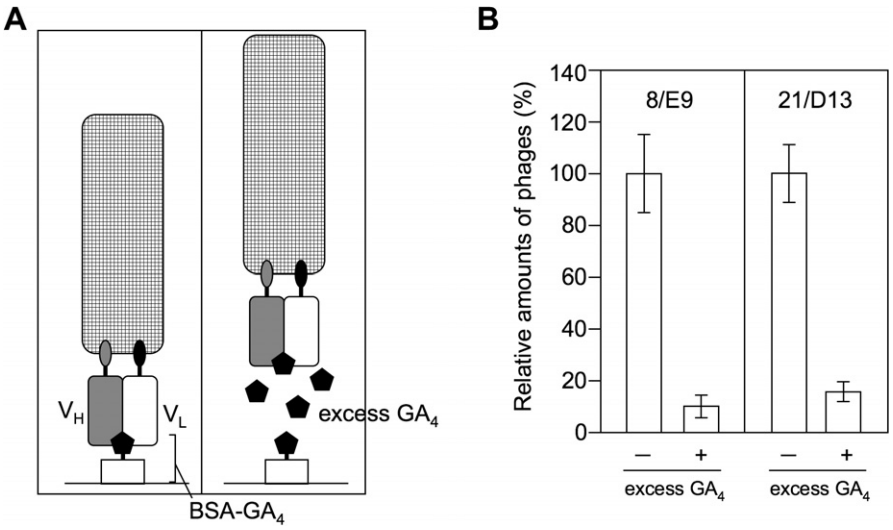

Fig. 1. Functional Fv formation by V_H and V_L simultaneously expressed on phage surface. Schematic drawing of the assay system is shown (A). V_H and V_L were displayed on a phage particle by expressing as a fusion with p9 and p7, respectively. The formation of Fv and its binding activity to GA_4 was assessed by ELISA (B). The excess GA_4 was used for the competitive ELISA at 0.1 mM.

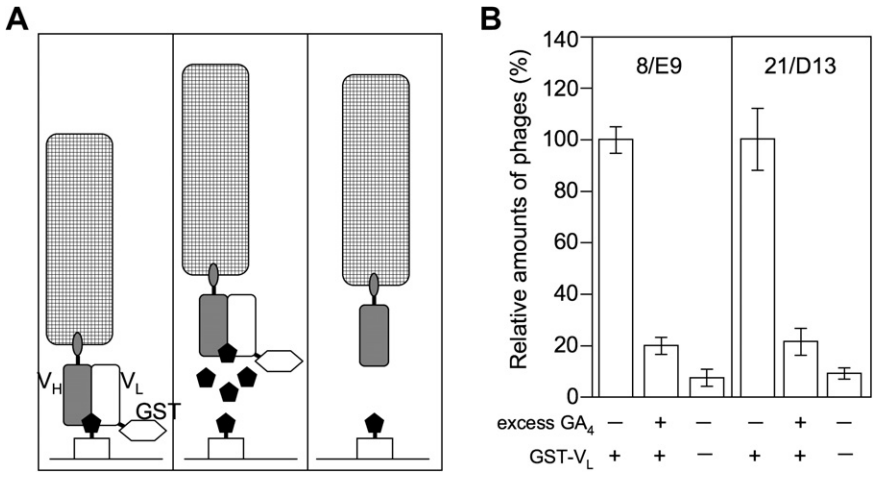

Fig. 2. Functional Fv formation by V_H and V_L separately expressed. Schematic drawing of the assay system is shown (A). V_H was displayed on a phage particle as a fusion with p3, and V_L was expressed as a soluble GST-tagged fusion protein. The formation of Fv and its binding activity to GA_4 was assessed by ELISA (B). The excess GA_4 was used for the competitive ELISA at 0.1 mM.

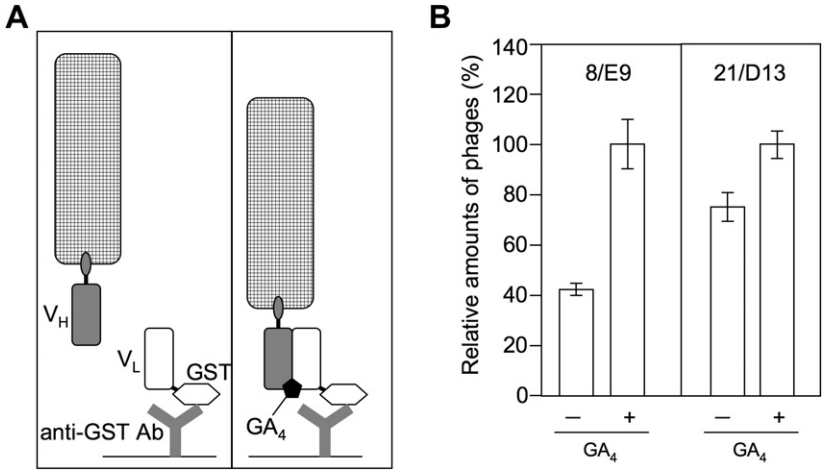

Fig. 3. GA_4-dependent interaction of V_H and V_L. Schematic drawing of the assay system is shown (A). V_H was displayed on a phage particle as a fusion with p3, and V_L was expressed as a soluble GST-tagged fusion protein. GA_4-dependent interaction of V_H and V_L was assessed by ELISA at 10 μM GA_4 (B).

which resides on the surface loop and has been successfully used for PCA [13,14]. Based on the fact that 8/E9 scFv, in which the C-terminus of V_H and N-terminus of $V_L$are connected with linker peptide, showed binding activity [7], we assumed that the C-terminus of V_H and the N-terminus of V_L are relatively close, and thus decided to fuse V_H to the N-terminus of CGFP (V_H-CGFP) and V_L to the C-terminus of NGFP (V_L-NGFP). Expression cassettes of these fusion proteins were constructed on a single plasmid vector and simultaneously produced in *E. coli*. When the protein expression was induced in the absence of GA_4, the bacteria showed scarce green light with an NIBA filter (Fig. 4B) and only yellowish intrinsic fluorescence with a GFP filter (Fig. 4E). Contrarily, when cultured in an inductive medium containing GA_4, bacteria showed stronger green fluorescence with an NIBA filter (Fig. 4C) and clear green fluorescence in addition to the yellowish intrinsic fluorescence with a GFP filter (Fig. 4F). Although the intensity of GFP fluorescence was not as high as that of *E. coli* expressing full-length GFP (Fig. 4D compared to Fig. 4C), the effect of GA_4 for reconstitution of the GFP molecule was apparent. Thus, the result demonstrates the feasibility of this system to detect GA_4 *in vivo* in the reductive *milieu* of a living organism.

Discussion

The novelty of this study resides in the idea that a ligand-dependent association of V_H and V_L could be applied to a detection system of the ligand *in vivo*. This notion is backed up by existing technologies to detect protein–protein interaction *in vivo* such as PCA. Because we already had scFv as recombinant antibodies against GA_4, we started by showing that the separately expressed V_H and V_L could form functional Fv. For this experiment we successfully used a split-Fv system, where V_H and V_L are displayed in proximity on phage. Although this system can also be used to monitor ligand-dependent association of V_H and V_L, V_L fragments examined in this study were not stably produced. Since the H and L chains assemble to form IgG upon production, it seems that V_L alone is less stable than Fv. This problem was overcome by producing V_L as a fusion protein with GST, which is an usual measure to produce proteins unstable in *E. coli*. The association between V_H and V_L of 8/E9 showed a clearer dependence on GA_4 than the V_H and V_L of 21/D13. There must be substantial affinity between V_H and V_L of 21/D13 in a non-liganded state. The crystal structure of GA_4-bound

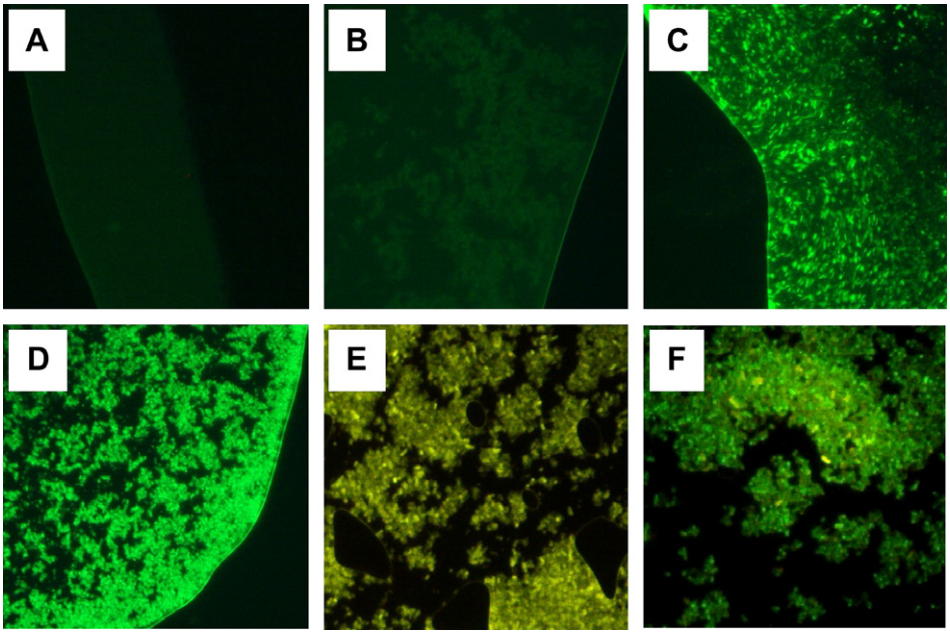

Fig. 4. Reconstitution of GFP by complementary association of NGFP and CGFP catalyzed by GA_4-dependent V_H/V_L interaction. *E. coli* in which NGFP-V_L and V_H-CGFP were inductively produced in the absence (B,E) or presence (C,F) of GA_4 (0.1 mM). (A) *E. coli* harboring an empty pETDuet-1 vector as a negative control. (D) *E. coli* in which full-length GFP was inductively produced as a positive control. (A–D) NIBA filter; (E,F) GFP filter.

Fab of 8/E9 antibody has been solved by X-ray diffraction [15]. The structure showed that GA_4 interacts with amino acids on V_H, but not on V_L, indicating the capability to develop OS-ELISA even when the apparent interaction of a ligand is only with V_H. It is unlikely that GA_4 acted as a glue to stick V_H and V_L together. It rather seems that V_H increased the affinity to V_L upon GA_4 binding by a conformational change, and that the resultant V_H/V_L-GA_4 complex became stable as a whole.

Despite the hurdle of substantial background in OS-ELISA, the capability of PCA to detect GA_4 *in vivo* was examined. Consequently, re-constitution of GFP catalyzed by a GA_4-dependent V_H/V_L interaction was clearly detected, which is the first case of GA_4 detection in a living organism. This is also the first example of PCA using V_H/V_L interaction mediated by a small molecule in *E. coli* cytosol, where Fvs harboring intra-molecular disulfide bonds are generally unstable because of its reducing condition. The GFP fluorescence in the absence of GA_4 was unexpectedly low. The background fluorescence level must depend on the protein levels of NGFP-V_L and V_H-CGFP. Thus, the application of this system to plants must include examination of their production levels and stabilities. There is another problem to be solved for *in planta* application, that is, low sensitivity of GA_4 detection. GA_4 was effective in the V_H/V_L interaction only above 10 μM both in OS-ELISA and PCA (data not shown). Although the localized concentrations of GA_4 in plants must be higher than the averaged value in organs or tissues determined by instrumental analysis, detection of GA_4 at 10 μM must be below the desirable level. Since an *in vivo* detection system in *E. coli* has been established in this study, a molecular evolution method is now feasible to obtain a suitable V_H and V_L pair, i.e., random error incorporation in V_H and V_L and screening of clones with low background and higher sensitivity in PCA.

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